R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways

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Background: The small GTPase R-Ras displays a less potent transforming activity than the closely related *Ras* oncogene products. Although R-Ras has been reported to interact with c-Raf1 and Ral-GDS *in vitro*, the pathways by which it exerts its effects on cellular proliferation are not known.

Results: Both Ras and R-Ras interact with phosphoinositide (PI) 3-kinase *in vitro*, and induce elevation of the levels of PI 3-kinase lipid products in intact cells. Unlike Ras, R-Ras does not activate Raf or mitogen-activated protein (MAP) kinase in cells. In co-transfection assays, the serine/threonine protein kinase PKB (or Akt) is effectively stimulated by R-Ras, Ras, mutants of Ras that activate PI 3-kinase but not other effectors, and activated forms of PI 3-kinase. Ras and R-Ras stimulate PKB/Akt through a non-autocrine mechanism that involves PI 3-kinase. The constitutive activation of PI 3-kinase alone is sufficient to activate PKB/Akt, but not the MAP kinase ERK or the stress-activated protein kinase, Jun N-terminal kinase. Transformation assays in fibroblasts suggest that PKB/Akt and Raf are part of distinct oncogenic signalling pathways.

Conclusion: Both the Raf–MAP kinase and PI 3-kinase–PKB/Akt pathways are activated by Ras, but only the PI 3-kinase-PKB/Akt pathway is activated by R-Ras. PI 3-kinase, and downstream targets such as PKB/Akt, are likely to be essential mediators of transformation induced by R-Ras. PI 3-kinase, as well as Raf, is thus implicated also in Ras transformation.

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Background

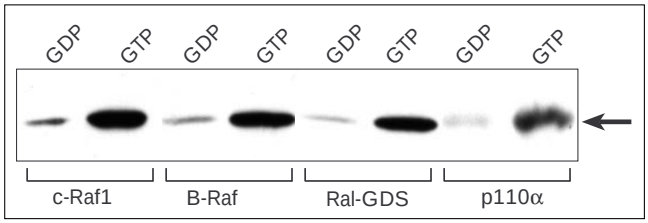
The R-Ras protein was originally identified because of its high level of homology to the H-*Ras*, K-*Ras* and N-*Ras* oncogene products, being about 55 % identical to each [1]. An R-Ras homologue, Ras2, has been identified in *Drosophila* [2]. R-Ras and the closely related protein TC21 form a distinct branch of the Ras subfamily [3]. Despite the considerable similarity to Ras, R-Ras has not been reported to be activated by mutation in human tumours. In tissue-culture experiments, activated mutants of R-Ras display considerably reduced transforming ability compared with activated Ras [4], and R-Ras has been reported to be unable to transform Rat-1 fibroblasts, although it can transform NIH 3T3 cells [4–6]. Like Ras, R-Ras interacts with the amino-terminal regulatory region of the Raf serine/threonine kinases, as does the related Ras antagonist Rap1 [7,8]. In short-term cellular assays, however, R-Ras has not been reported to induce significant activation of Raf or the extracellular signal-regulated kinase (ERK) subfamily of the mitogen-activated protein kinases (MAP kinases), although some, but not all, R-Ras transformed cells show partial activation of ERK [6]. R-Ras also interacts with the putative Ras effector Ral-GDS and related exchange factors for the Ras-related Ral proteins [9,10]; however, R-Ras is not able to activate Ral-GDS in intact cells [11]. In terms of upstream regulatory components, R-Ras interacts with the GTPase-activating proteins for Ras, $p120^{GAP}$ and neurofibromin, which stimulate R-Ras GTP hydrolysis [7,12], but is not stimulated by Sos, the guanine nucleotide exchange factor for Ras [13]. The extracellular stimuli that control the activation state of R-Ras are unknown.

We have reported previously that GTP-bound Ras interacts directly with the catalytic subunit of phosphoinositide (PI) 3-kinase, p110α, to induce its activation, particularly in synergy with other signalling pathways [14–16]. It has also been shown that efficient activation of PI 3-kinase by platelet-derived growth factor (PDGF) requires Ras function [17]. PI 3-kinases are one family of target enzymes, or effectors, of Ras, therefore, in addition to the Raf kinases and Ral-GDS, the nucleotide exchange factors for the Ras-related Ral proteins [18]. The role of PI 3-kinase in the transforming activity of oncogenic Ras is currently unknown, although PI 3-kinase activity has been reported to be required for the growth of normal fibroblasts [19]. A number of signalling systems have been implicated downstream of PI 3-kinase, including the Rho-related Ras superfamily protein Rac [20] and the serine/threonine kinases $p70^{S6K}$ [21], protein kinase B (PKB or Akt) [22,23] and novel [24] and atypical [25] isoforms of protein kinase C.

PKB/Akt was identified by its similarity to protein kinases A and C [26,27], and was shown to be the product of the cellular homologue of the *v-Akt* retroviral oncogene [28]. The treatment of cells with growth factors such as insulin or PDGF activates PKB/Akt through a mechanism that requires PI 3-kinase activity and an intact pleckstrin homology (PH) domain. The activation of PI 3-kinase has been shown recently to be sufficient to activate PKB/Akt in a co-transfection assay [29].

In this study, we have set out to investigate the pathways used by R-Ras in regulating cell proliferation and transformation. We show that R-Ras does not activate Raf efficiently, but does activate PI 3-kinase and PKB/Akt. This pathway appears to be an important growth stimulatory mechanism used equally well by both Ras and R-Ras.

Figure 1



R-Ras binds to c-Raf1, B-Raf, Ral-GDS and PI 3-kinase (p110α) in a GTP-dependent manner *in vitro*. The Ras-binding domains of c-Raf1 [53], B-Raf, Ral-GDS and p110α [15] were expressed as GST-fusion proteins and immobilized on glutathione beads. Purified R-Ras protein was loaded with either GDP or GTP and incubated with the beads. After washing, the bound R-Ras protein was electrophoresed on a 15 % SDS gel and detected with an anti-R-Ras antibody by western blotting. The R-Ras band is indicated by an arrow.

Results

Interaction of R-Ras with effectors *in vitro*

To investigate possible signalling pathways used by R-Ras, we studied the ability of R-Ras to interact with effector molecules that have been shown to interact with Ras. Purified R-Ras was loaded with GTP or GDP, then mixed with glutathione S-transferase (GST) fusion proteins that included the Ras binding sites of c-Raf1, B-Raf, Ral-GDS and PI 3-kinase p110α. R-Ras retained on glutathione agarose in a complex with the effectors was detected by western blotting. As shown in Figure 1, R-Ras interacted in a GTP-dependent manner with each of these effectors. In each case, the interaction was approximately comparable to that with Ras (data not shown). As both Ras and R-Ras interact with the fusion protein including amino acids 133–314 of p110α, it is likely that both interact with PI 3-kinase at the same, or similar, sites. Indeed competition between the two for binding to this PI 3-kinase fragment was observed *in vitro* (data not shown).

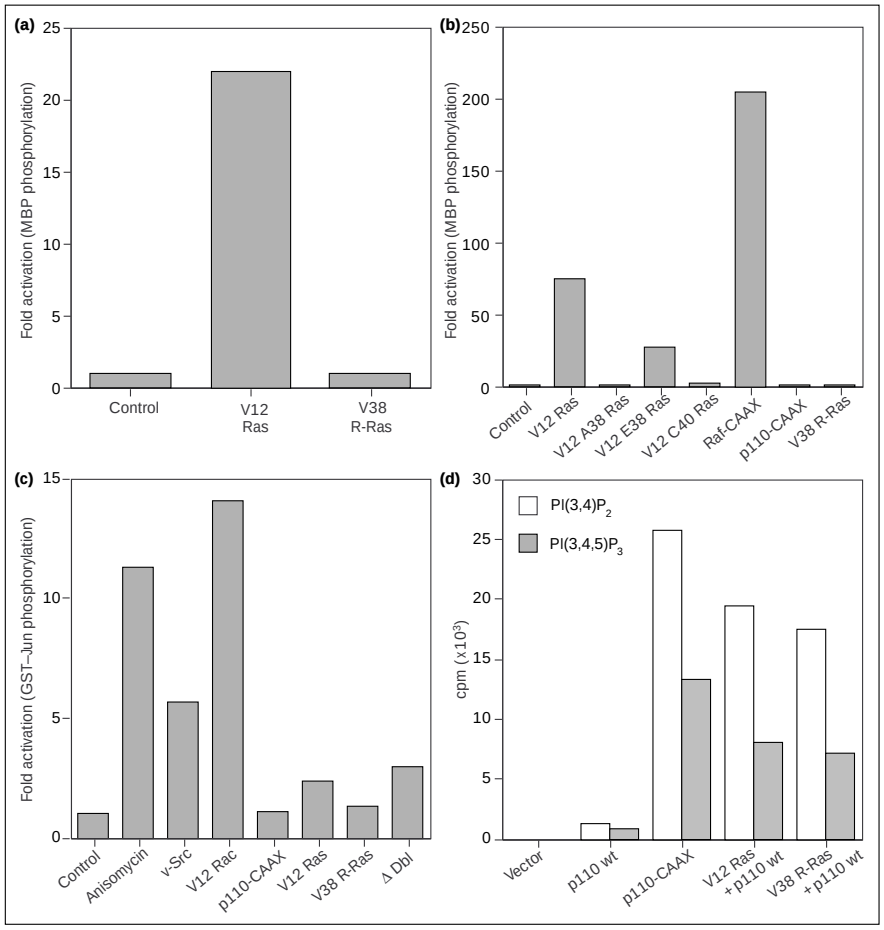
Regulation of effectors by R-Ras in intact cells

The ability of R-Ras to activate effector enzymes was tested in whole cells. COS cells were transiently transfected with plasmids encoding activated mutants of R-Ras (V38, containing a valine at position 38) or Ras (V12, the equivalent to V38 in R-Ras), along with epitope-tagged c-Raf1. Raf was immunoprecipitated from lysates of these cells, and its activity determined by measuring its ability to activate the MAP kinase kinase MEK. As shown in Figure 2a, whereas activated V12 Ras efficiently stimulated Raf, V38 R-Ras was completely inactive in this assay. The ability of R-Ras, Ras and other signalling molecules to activate ERK2 MAP kinase was also tested by co-transfection with an epitope-tagged ERK2; the activity of the transfected ERK2 was measured following immunoprecipitation (Fig. 2b). V38 R-Ras did not activate ERK2 in this assay, whereas V12 Ras and activated Raf (Raf-CAAX, which is localized to the membrane by the addition of a prenylation sequence) activated ERK2 strongly. p110-CAAX, a strongly activated membrane-localized form of PI 3-kinase, failed to activate ERK2. In addition, some mutants of the effector site of Ras were assayed. ERK2 was not activated by V12 A38 Ras (a total loss-of-function mutant which is unable to bind to any effectors) or by V12 C40 Ras (a mutant which is unable to bind to Raf or Ral-GDS, but which interacts with PI 3-kinase, albeit not as strongly as wild-type; P.R-V., P.H. Warne and J.D., unpublished observations). ERK2 was activated by V12 E38 Ras, a mutant which is able to bind to Raf, although not as efficiently as wild-type, but which does not bind to PI 3-kinase or Ral-GDS (P.R-V., P.H. Warne and J.D., unpublished observations). We conclude that the ERK2 MAP kinase can be activated by an interaction between Ras and Raf, but not by an interaction between R-Ras and Raf, and that ERK2 activation is not caused by the activation of PI 3-kinase.

As well as Raf and ERK2, we studied the ability of R-Ras to activate the stress-activated kinase Jun N-terminal kinase (JNK) (Fig. 2c). As reported previously [30,31], V12 Rac, v-Src and anisomycin stimulated JNK strongly, whereas V12 Ras activated JNK only weakly. Similar results were observed for both the p54 and p46 forms of JNK. An activated form of Dbl, a guanine nucleotide exchange factor for Rho family proteins, was also able to stimulate JNK, presumably acting through endogenous Cdc42 or Rac. R-Ras was completely unable to stimulate JNK in this assay, as was activated PI 3-kinase. This result was somewhat unexpected, as study of the actin cytoskeleton in fibroblasts has indicated that PI 3-kinase appears to be able to activate Rac, an activator of JNK, in that system (see Discussion).

The ability of R-Ras to regulate PI 3-kinase was investigated by measuring the levels of the lipids phosphatidylinositol (3,4) P_2 ($PI(3,4)P_2$) and phosphatidylinositol (3,4,5) P_3 (PIP_3) in transiently transfected COS cells. Upon transient transfection into COS cells, activated Ras synergizes strongly with wild-type p110α to give increased levels of

Figure 2



R-Ras activates PI 3-kinase *in vivo*, but not Raf, MAP kinase or JNK. (a) Raf activity was measured in COS-7 cells by co-transfecting V12 Ras or V38 R-Ras with Myc-tagged c-Raf1. After 48 h, Raf was immunoprecipitated and assayed in a coupled assay for its ability to activate MEK. (b) COS-7 cells were transfected with 1 μg Myc-tagged MAP kinase (p42 ERK2) and 1 μg of the indicated plasmids. After 48 h, ERK2 was immunoprecipitated with the 9E10 antibody. Activity was determined using MBP (myelin basic protein) as a substrate. Reactions were electrophoresed on a 15 % SDS gel and phosphorylated MBP was quantified with a phosphoimager. (c) COS-7 cells were transfected with 1 μg hemagglutinin (HA)-tagged JNK (p54 SAPK) and 1 μg of the indicated plasmids. After 48 h, JNK was immunoprecipitated with the 12CA5 antibody. Activity was determined using GST–Jun as a substrate. Reactions were run on a 15 % SDS gel and quantified with a phosphoimager. (d) 48 h after transfection with the indicated plasmids, COS-7 cells were labelled with 0.25 mCi ^{32}P-orthophosphate for 5 h. Lipids were extracted and analysed by HPLC as described in [14].

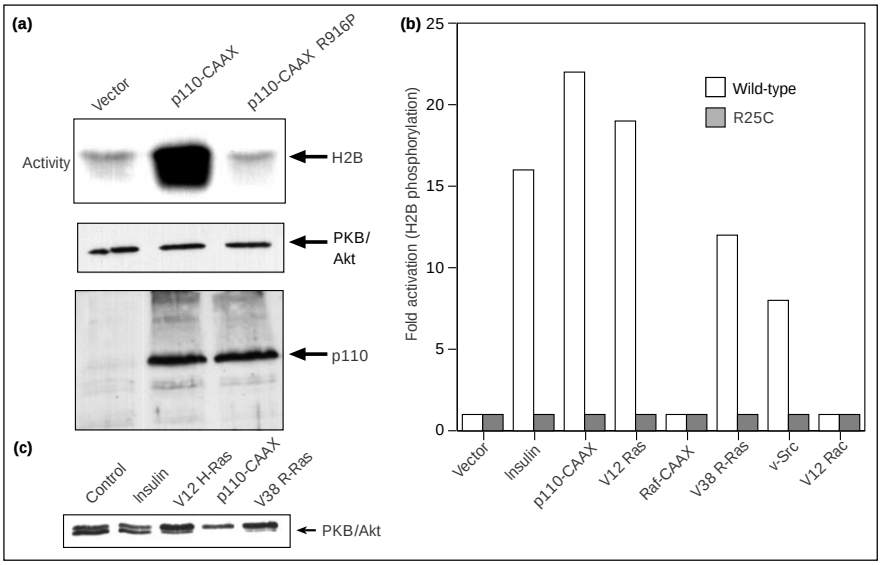
the PI 3-kinase product lipids PI(3,4)P_2 and PIP_3 [14,15]. As shown in Figure 2d, R-Ras was also able to elevate the levels of PI(3,4)P_2 and PIP_3 in this way. In this assay, p110α alone showed constitutive activation when it was localized to the plasma membrane by a carboxy-terminal farnesylation signal from H-Ras (CAAX; [32]). In the absence of overexpressed wild-type p110α, both activated Ras and R-Ras give only modestly elevated levels of PI(3,4)P_2 and PIP_3 ([14] and data not shown); this is presumably due to the relatively small fraction of cells expressing the transfected genes. Thus, R-Ras is able to activate the PI 3-kinase pathway in cells, with an efficiency similar to that of Ras.

The effect of activating PI 3-kinase on the activity of PKB/Akt was investigated by immunoprecipitating PKB/Akt tagged with a hemagglutinin (HA) epitope [23] from transiently transfected COS cells, and assaying for its ability to phosphorylate histone 2B [22]. p110-CAAX, but not a kinase-inactive p110 mutant, caused a robust activation of PKB/Akt (Fig. 3a). We also used the same assay on NIH 3T3 cells overexpressing the insulin receptor (A14 cells; Fig. 3b): PKB/Akt was stimulated 22-fold by p110-CAAX, 16-fold by insulin treatment, 13-fold by activated V38 R-Ras alone, 19-fold by activated V12 Ras alone, and 8-fold by v-Src (which has also been shown to elevate PIP_3 levels [14]). However, activated Raf and the Rho-related GTPase Rac were unable to influence PKB/Akt activity. A point mutation in the PH domain of PKB/Akt, R25C (with an arginine residue replacing the cysteine at position 25 [22]), completely blocked its activation by all these stimuli. It would appear, therefore, that all the stimuli capable of activating PI 3-kinase, including R-Ras, are able to activate PKB/Akt. Immunoblotting of PKB/Akt from A14 cells showed that there was a marked shift in apparent molecular weight in cells expressing PI 3-kinase, Ras or R-Ras, probably indicating an alteration in the phosphorylation state of PKB/Akt (Fig. 3c).

R-Ras activation of PKB/Akt is through a non-autocrine mechanism involving PI 3-kinase

A problem in interpreting data from transient transfection experiments is that the assay is performed over a sufficiently long period for autocrine production of growth

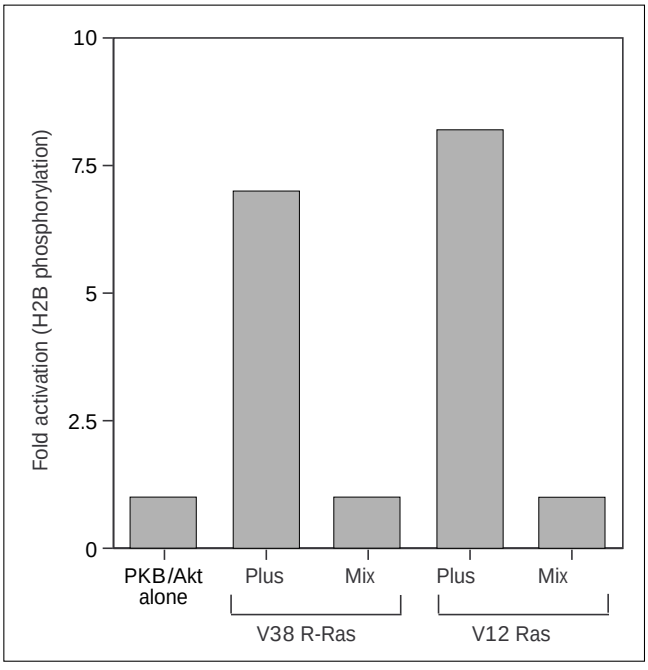
Figure 3



Activation of PKB/Akt by R-Ras, H-Ras or membrane-targeted PI 3-kinase. **(a)** PKB/Akt activity was measured in COS-7 cells co-transfected with vector alone, p110-CAAX or the kinase-inactive version p110-CAAX R916P (with an arginine residue replacing the proline at position 916; [52]) (upper panel). Western-blot analysis of the immunoprecipitations showed equal expression levels of PKB/Akt (middle panel). The expression of active and inactive p110-CAAX was compared in total lysates (lower panel). **(b)** A14 cells were transfected with HA-PKB/Akt (wild-type or the R25C mutant) plus the indicated plasmids. Some cells were treated with 10 μg ml^{-1} insulin for 2 min. PKB/Akt activity was determined as described in Materials and methods. **(c)** HA-PKB/Akt was immunoprecipitated from A14 cells co-transfected with the indicated plasmids and separated on a 15 % SDS-PAGE gel, which was then probed with an anti-PKB/Akt antibody.

factors to occur, which might contribute to the activation of downstream enzymes [33]. The activation of PKB/Akt by R-Ras and Ras may therefore be mediated by the production of autocrine growth factors which activate PI 3-kinase. To investigate this question, R-Ras or Ras were either co-transfected with epitope-tagged PKB/Akt into COS cells, or R-Ras or Ras and PKB/Akt were transfected into separate populations of cells which were subsequently mixed and cultured together. R-Ras and Ras activated PKB/Akt only when they were present in the same cells, and not when they were expressed in neighbouring cells in the same culture (Fig. 4). It is unlikely, therefore, that R-Ras or Ras activates PKB/Akt by an autocrine mechanism.

Figure 4

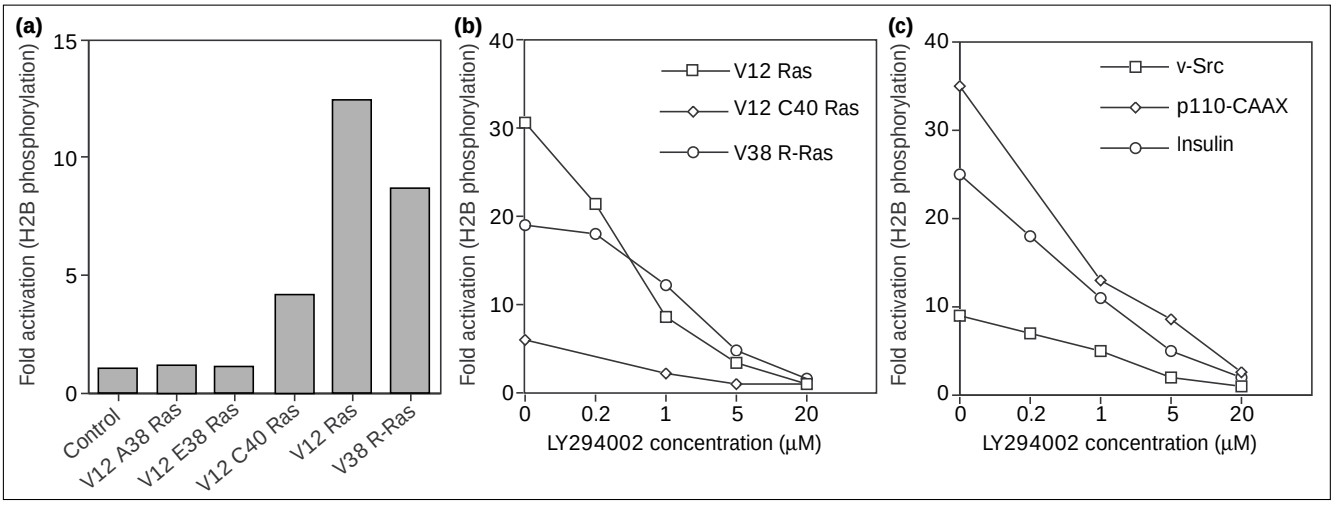


The activation of PKB/Akt by R-Ras and H-Ras is not autocrine. COS-7 cells were co-transfected with HA-PKB/Akt and V38 R-Ras or V12 Ras (Plus), or R-Ras, Ras and HA-PKB/Akt were transfected into separate pools of cells which were subsequently mixed (Mix). Kinase activity was determined as described in Materials and methods.

As shown in Figure 3, there was a strong correlation between activation of PI 3-kinase and activation of PKB/Akt. To investigate this observation further, partial loss-of-function effector mutants in Ras were studied. PKB/Akt was activated by V12 C40 Ras, although more weakly than by V12 Ras or V38 R-Ras, whereas V12 E38 Ras and V12 A38 Ras (which is entirely inactive) both failed to activate PKB/Akt (Fig. 5a).

The connection between PI 3-kinase activation and PKB/Akt activation was tested directly using PI 3-kinase inhibitors. To investigate whether PI 3-kinase activity was required for R-Ras and Ras to stimulate PKB/Akt, the well-characterized PI 3-kinase inhibitor LY294002 was used [34]. LY294002 inhibited the activation of PKB/Akt by V38 R-Ras and V12 Ras in A14 cells, with approximately 1 μM being required for 50 % inhibition (Fig. 5b); this value is similar to the half-maximal inhibitory concentration (IC_{50}) for PI 3-kinase [34]. A similar sensitivity to LY294002 was observed for the activation of PKB/Akt by V12 C40 Ras, p110-CAAX, v-Src and insulin (Fig. 5b,c). A second PI 3-kinase inhibitor, wortmannin, also inhibited this PKB/Akt activation at 100 nM (data not shown).

Figure 5



R-Ras and Ras activate PKB/Akt *via* PI 3-kinase. PKB/Akt activity was measured in COS-7 (a,b) or A14 (c) cells after co-transfection with the indicated plasmids. In (b,c), cells were incubated with increasing concentrations of LY294002 or the carrier DMSO for 2 h prior to lysis. Insulin treatment (10 μg ml^{-1}) was carried out for 2 min.

Neither inhibitor is completely specific for PI 3-kinase, but, at the concentrations used, LY294002 and wortmannin are the best available means for inhibiting PI 3-kinase.

The role of the PKB/Akt pathway in transformation

The data presented here indicate that PKB/Akt acts as a target of PI 3-kinase activity and as part of the effector signalling pathways of both Ras and R-Ras. By contrast, Ras also uses the Raf–MAP kinase pathway, but R-Ras does not. To investigate the potential contribution of PKB/Akt to the transformation of cultured fibroblasts by R-Ras and Ras, NIH 3T3 cells were transfected with a construct encoding a PKB/Akt which had been activated by fusion to a retroviral gag sequence [23]. This activated PKB/Akt alone was unable to transform these cells, as were the V12 C40 Ras and V12 E38 Ras mutants (which activate PI 3-kinase and Raf, respectively), but PKB/Akt synergized with V12 E38 Ras to give transformation (Table 1). PKB/Akt did not synergize with V12 G37 Ras (which activates Ral-GDS) or with V12 C40 Ras, presumably because it acts on the same pathway. Thus PKB/Akt, although a very weak oncogene by itself, can provide a transforming signal which complements that of the Raf pathway.

Table 1

Transformation of NIH/3T3 cells by Ras mutants and gag-PKB/Akt.

Plasmids	Plasmid concentration	Relative number of foci
V12 Ras	50 ng	0.3
	500 ng	1
V38 R-Ras	50 ng	0.06
	500 ng	0.4
	2 μg	1
v-Akt		0.03
v-Akt + G37 Ras		0
v-Akt + E38 Ras		0.29
v-Akt + C40 Ras		0
G37 Ras		0
E38 Ras		0.03
C40 Ras		0
E38 Ras + G37 Ras		0
E38 Ras + C40 Ras		0

Plasmids encoding V12 Ras, V38 R-Ras, other Ras mutants (in a V12 background) and gag-PKB/Akt (v-Akt) were transfected into NIH/3T3 cells either alone or in combinations. Focus-formation assays were performed over two weeks. Numbers represent data from at least two independent experiments.

Discussion

Here we have shown that the Ras subfamily member R-Ras activates PI 3-kinase and PKB/Akt in cells with an efficiency similar to that of Ras, but is extremely inefficient at activating Raf, despite interacting strongly with Raf *in vitro*. The reason for the poor ability of R-Ras to activate Raf is not clear, but may be due to differences in the intracellular localization of Ras and R-Ras. Ras is found exclusively at the plasma membrane, but the localization of R-Ras has not been reported, and may be on intracellular membranes. The ability of Ras to bring c-Raf1 to the plasma membrane, where it may be acted on by other components of the activation machinery, is thought to be a crucial part of its function [35,36]. It has been reported that some clones of NIH 3T3 cells transformed by R-Ras exhibit partial activation of ERK2 MAP kinase [6]; as R-Ras does not activate Raf or ERK2 in transient assays in NIH 3T3 or COS cells, it is

possible that this activation of ERK2 is the result of other causes and has been selected for during the transformation assay.

The exact mechanism involved in the activation of PI 3-kinase by Ras and R-Ras has not been elucidated, although it is clear that purified, post-translationally modified Ras–GTP can activate purified PI 3-kinase in a liposome system *in vitro* [15]. There may not be a requirement for this interaction to occur at the plasma membrane rather than any other membrane. As R-Ras activates both PI 3-kinase and its downstream target PKB/Akt, the second-messenger 3′ phosphorylated phosphoinositides generated may be as available to PKB/Akt as are the lipids generated in response to Ras or p110-CAAX expression. The data shown in Figure 4 indicate that the mechanism involved is likely to be the direct stimulation of PI 3-kinase by interaction with Ras and R-Ras proteins, rather than through a pathway mediated by autocrine growth factors.

The activation of PKB/Akt by Ras and membrane-localized p110 has been reported recently [29]. In co-transfection experiments described in that report and here, activation of PI 3-kinase appears sufficient to stimulate PKB/Akt. However, in the function of normal cellular growth-factor signalling pathways, it is likely that a second signal in addition to PI 3-kinase is required: for example, there is considerable discrepancy between the ability of growth factors to activate PI 3-kinase and PKB/Akt, although PI 3-kinase is clearly required [22,23,37]. PIP_3, and to a lesser extent $PI(3,4)P_2$, have been reported to bind specifically to the PH domain of PKB/Akt [38]; the exact mechanism whereby this interaction can be sufficient to cause PKB/Akt activation in whole cells is unclear, but may involve presentation of the enzyme to other kinases, possibly at the plasma membrane, alteration of the dimerization state of PKB/Akt, or direct allosteric regulation of its kinase activity [22,37,39,40]. The only downstream target of PKB/Akt identified to date is glycogen synthase kinase-3 [41], but others doubtless exist. The frequent overexpression of the closely related Akt2/PKBβ in ovarian and pancreatic cancer [42,43] lends weight to the idea that pathways controlled by PKB/Akt are important in human tumourigenesis. Our demonstration that R-Ras is as able as Ras to activate PKB/Akt shows that this pathway may be an important component of the signalling machinery used by R-Ras, as well as Ras, in cellular transformation.

As R-Ras can activate PI 3-kinase and PKB/Akt, is it involved in PKB/Akt activation induced by growth factors? The dominant-negative N17 Ras mutant is able to inhibit (by 60–70 %) the activation of PKB/Akt by PDGF, insulin and epidermal growth factor (EGF), suggesting that Ras is of major importance in this pathway ([22] and our unpublished observations). No effect of expressing dominant-negative R-Ras on activation of PKB/Akt by these growth factors has yet been seen. However, the growth stimuli that normally activate R-Ras are currently unknown. It is perhaps likely that R-Ras responds to different upstream signals to Ras, but at present these remain elusive. It is unlikely that the ability of R-Ras to activate PI 3-kinase and PKB/Akt is simply the result of targeting PI 3-kinase to its substrates in any membrane, as another Ras subfamily protein, Rap1a, is unable to activate either PI 3-kinase or Akt in intact cells ([15] and our unpublished observations).

Unlike Klippel *et al.* [29], we were not able to see any ability of activated PI 3-kinase to stimulate the stress-activated kinase JNK in co-transfection assays. The reason for this discrepancy is not clear. Ras shows modest activation of JNK, as reported previously by others [30,31], but R-Ras does not. Ras-induced activation of JNK could be mediated by direct interaction of Ras with MEKK (MAP kinase kinase kinase; [44]) or with JNK itself [45]; it is not known whether R-Ras interacts with these targets. The ability of Ras and R-Ras to activate PI 3-kinase similarly, but of only Ras to activate JNK, indicates that it is unlikely that the activation of JNK by Ras is mediated by PI 3-kinase. Moreover, PI 3-kinase inhibitors such as wortmannin and LY294002 are not able to block the activation of JNK induced by Ras, Src or growth factors; on the contrary, treatment of cells with these inhibitors alone leads to activation of JNK (our unpublished results and [46]). It is possible that PI 3-kinase may contribute in some way to JNK activation, but that other signalling inputs are required. For example, it has been reported recently that the adaptor protein Nck may be involved in linking receptor tyrosine kinases to the activation of PAK (p21-activated kinase) and JNK [47].

The biological function of R-Ras has been obscure. Microinjection experiments failed to find an effect of R-Ras protein on DNA synthesis or cell morphology, although it is possible that R-Ras function was compromized by defective post-translational modification of the bacterially expressed protein [7]. Activated mutant forms of R-Ras have been found to induce transformation of NIH 3T3 fibroblasts, but not Rat-1 fibroblasts [4–6]. R-Ras transformation differs from Ras transformation in that R-Ras-transformed NIH 3T3 cells do not display the refractile morphology of Ras-transformed cells [6]. This is perhaps surprising, given that the PI 3-kinase pathway is thought to have a major short-term impact on the actin cytoskeleton; however, the events required for long-term morphological transformation are unknown, and certainly require more than just PI 3-kinase activation (P.R-V. and J.D., unpublished results). When co-expressed with Myc, R-Ras will give morphological transformation [6]. Presumably, Ras is able to provide all the signals necessary to cause morphological transformation, whereas R-Ras provides only some. R-Ras synergizes strongly with Raf in the transformation of NIH 3T3 cells, indicating that R-Ras lacks the ability to activate the Raf

pathway [5]. From the transformation assays reported here, it appear that the Raf–MAP kinase and the PI 3-kinase–PKB/Akt pathways act synergistically in the transformation of murine fibroblasts. It is clear that activation of the PI 3-kinase–PKB/Akt pathway alone is not sufficient to give efficient transformation of NIH 3T3 cells, even though it synergizes strongly with the Raf pathway.

Other aspects of R-Ras function have been reported. R-Ras may interact with Bcl-2, an important regulator of programmed cell death, and has been suggested to promote apoptosis in IL-3 dependent cells [48,49]. However, the significance of this observation remains controversial. In addition, R-Ras has recently been reported to promote integrin activation [50]; the mechanism involved has not been established, but could involve PI 3-kinase [51]. The significance of this to R-Ras-induced transformation is unclear. The data presented in this paper establish, for the first time, an effector pathway used by R-Ras in cells — the PI 3-kinase–PKB/Akt pathway. Future work will address other signalling mechanisms that might be used by R-Ras *in vivo*, and the regulatory mechanisms that act upstream of R-Ras.

Conclusions

The potent transforming potential of Ras proteins has been well characterized. By contrast, the closely related GTPase R-Ras is less well understood, despite being expressed ubiquitously in a wide range of species. Ras interacts with a number of target enzymes *in vitro* and has been shown to activate Raf and PI 3-kinase *in vivo*. R-Ras is able to interact with the same effectors, but can only activate PI 3-kinase, and not Raf, in whole cells. Both Ras and R-Ras can activate PKB/Akt, a serine/threonine kinase downstream of PI 3-kinase that itself has transforming potential, through a non-autocrine mechanism. Constitutive activation of PI 3-kinase alone is sufficient to activate PKB/Akt, but not the MAP kinase ERK or the stress-activated protein kinase JNK. Transformation assays in fibroblasts show that PKB/Akt and Raf are part of distinct oncogenic signalling pathways which act synergistically. PI 3-kinase and downstream targets such as PKB/Akt are likely to be essential mediators of R-Ras-induced transformation. This work also emphasizes the likely importance of PI 3-kinase in Ras transformation.

Materials and methods

Plasmids, proteins and antibodies

The Ras effector mutants were made in a V12 background in pSG5 (P.R-V., P.H. Warne and J.D., unpublished observations). p110α-CAAX and p110α-CAAX R916 [52] in pSG5 will be described elsewhere (S.W. and J.D, unpublished observations). HA-PKB/Akt (wild-type and R25C) and gag-PKB/Akt were a generous gift from B. Burgering and P.Coffer [23]. ΔDbl was kindly provided by M. Olson and A. Hall.

GST-fusion proteins encompassing the Ras binding sites of c-Raf , Ral-GDS and p110α are described in [14,15,53] and P.R-V., P.H.Warne and J.D. unpublished observations). Amino acids 130–310 of quail B-Raf [54] were cloned into the pGEX (Pharmacia) vector for expression as a GST fusion protein. The Glu-tagged R-Ras baculovirus vector was obtained from F. McCormick (Onyx). R-Ras was purified from Sf9 cells with an antibody column. The polyclonal antiserum recognizing p110 (kindly provided by L. Claeson-Welsh and R. Hooshman-Rad) was raised against the carboxy-terminal 14 amino acids of bovine p110α.

R-Ras binding assays in vitro

50 μl of binding buffer (50 mM Hepes pH7.5, 50 mM NaCl, 5 mM $MgCl_2$, 5 mg ml^{-1} BSA) containing 0.5 μM R-Ras and 200 μM GDP or GTP was incubated at 37 °C for 5 min. Magnesium chloride was added to 10 mM followed by 20 μl of 1:1 suspension of glutathione agarose beads bound to GST-fusion protein. The volume was made up to 200 μl with phosphate buffered saline (PBS), 5 mM $MgCl_2$, 1 % Triton X-100 ,and the mixture was rotated at 4 °C for 2 h. The beads were then washed consecutively in 1 ml PBS containing 1 % Triton X-100, 5 mM $MgCl_2$, then in 1 ml 100 mM Tris pH 7.5, 500 mM LiCl, 5 mM $MgCl_2$, and then in 1 ml of 50 mM Tris pH7.5, 100 mM NaCl, 5 mM $MgCl_2$. The beads were finally resuspended in SDS PAGE sample buffer. Bound R-Ras was detected by immunoblotting using an anti-R-Ras antibody (Santa-Cruz).

Kinase assays

HA-PKB/Akt activity was assayed 48 h after transfection of COS-7 or A14 cells with the lipofectamine kit from Gibco/BRL. HA-PKB/Akt in the cells was immunoprecipitated with the 12CA5 antibody. The immunoprecipitates were washed and assays were performed as in [23], except that histone 2B was used as a substrate. HA-PKB/Akt was detected on western blots with an polyclonal antibody directed against the carboxyl terminus of PKB/Akt [22]. Raf activity was assayed by co-transfection of a myc-tagged c-Raf1 construct and performing a coupled MEK/ERK2 kinase assay on 9E10 immunoprecipitates as described [55]. Myc-tagged p42 was immunoprecipitated from transfected cells with 9E10 antibody and MBP phosphorylation was determined as described [55]. HA-JNK (p54 SAPK) was immunoprecipitated with 12CA5 antibody. Activity was determined as for MAPK, using GST-Jun as a substrate.

Transformation assays in NIH/3T3 cells

Low passage NIH/3T3 cells were seeded at 10^5 per well in 6-well dishes the day before lipofection with 0.5–2.5 μg of each plasmid, apart from V12 Ras and V38 R-Ras where the indicated concentrations were used (Table 1). Two days later, the cells were transferred to 10 cm plates. After reaching confluency, they were kept in DME medium containing 5 % calf serum for 2 weeks, after which they were stained with O.5 % crystal violet to visualize foci. On average, about 150 foci were obtained with 10 ng of V12 Ras.

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Erratum

R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways

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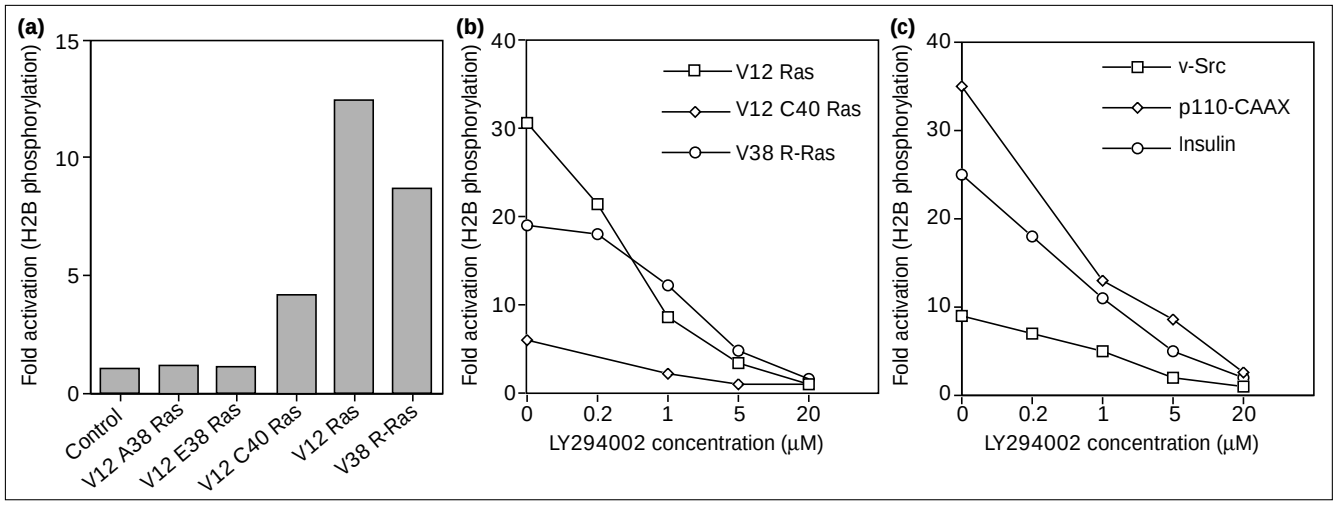
In this paper, which appeared in the 1 January 1997 issue of *Current Biology*, the labelling on the *x* axis of Figure 5a was inadvertently omitted. The correct version of the figure can be seen below.

Figure 5



R-Ras and Ras activate PKB/Akt *via* PI 3-kinase. PKB/Akt activity was measured in COS-7 (a,b) or A14 (c) cells after co-transfection with the indicated plasmids. In (b,c), cells were incubated with increasing concentrations of LY294002 or the carrier DMSO for 2 h prior to lysis. Insulin treatment (10 μg ml^{-1}) was carried out for 2 min.